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Cryopreservation of Mammalian Oocytes

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Summary

Two methods for the cryopreservation of mammalian oocytes are described. One method uses a relatively low concentration of the cryoprotectant propanediol plus sucrose and requires controlled-rate cooling equipment to achieve a slow cooling rate. Such a method has produced live births from cryopreserved human oocytes. The second method described employs a high concentration of the cryoprotectant dimethyl sulfoxide plus a low concentration of polyethylene glycol. This method involves cooling by plunging standard straws into liquid nitrogen vapor, hence avoiding the need for specialized equipment, but requires technical ability to manipulate the oocytes quickly in the highly concentrated cryoprotectant solutions. Murine oocytes vitrified, using this technique, have resulted in live births.

Key Words: Egg; human oocyte; oocyte; slow-cool; vitrification.

1. Introduction

Storage of unfertilized oocytes has numerous applications. In the treatment of human infertility these applications include storage of oocytes collected prior to receipt of cancer treatments or premature ovarian failure, banking of excess oocytes produced as a result of in vitro fertilization treatments, and donation of oocytes to other people (storage giving the added advantage of allowing time to screen donors for disease and in some countries, e.g., Taiwan, donation of oocytes but not embryos is authorized). Oocyte cryopreservation could also serve as a means of delaying child bearing to an age when natural fertility and/or oocyte quality has declined. Applications in animal management include the preservation of precious strains, the ability to restock following outbreaks of diseases such as foot and mouth, the preservation of genetically modified strains, thereby reducing the cost of continuous breeding and avoiding problems of genetic drift, and the preservation of endangered species. Oocyte preservation

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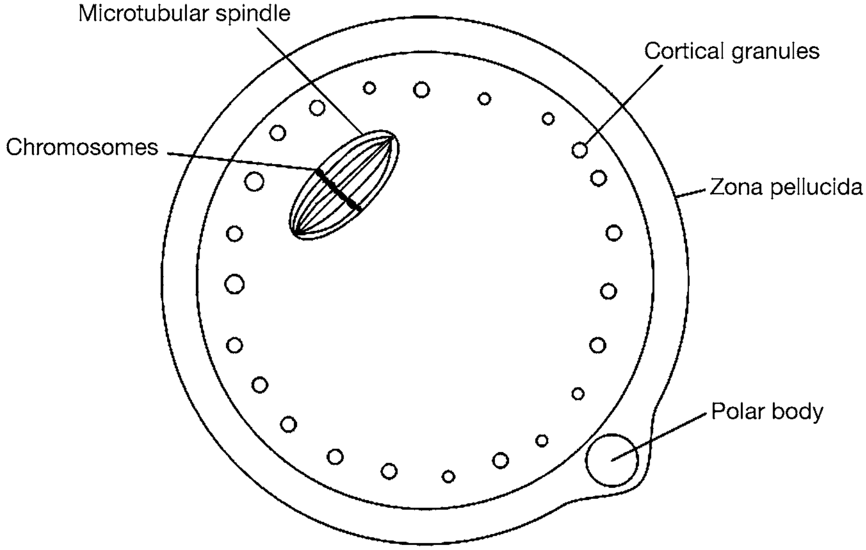

Fig. 1. Schematic representation of a mature mammalian oocyte.

is considered preferable to embryo preservation in humans because ethical concerns are fewer for unfertilized gametes. Furthermore, in some countries cryopreservation of human embryos is banned or is strictly limited. In animal management, oocyte cryopreservation gives greater flexibility in breeding programs than embryo cryopreservation.

In 1977, the first live births from cryopreserved ovulated mammalian oocytes were reported in mice *(1)*. Since then births resulting from cryopreserved oocytes have been reported in a number of species including rabbits *(2)*, cows *(3)*, horses *(4)*, and humans *(5)*. However, in most species success rates are markedly poorer than for cryopreserved embryos.

Oocytes are proving to be very difficult cells to cryopreserve for a number of reasons. The oocyte is a large single cell with low permeability to water. This means that it has a tendency to retain water when cooled and, if this forms intracellular ice, damage to the cell results. Permeability of oocytes varies between species, strains, and maturational status of the oocyte. The oocyte is also a short-lived cell that must undergo fertilization in order for it to continue to survive and develop. For fertilization to occur naturally, the oocyte must retain integrity of a number of its unique structural features. These include the zona pellucida, the cortical granules, and the microtubular spindle (*see* **Fig. 1**). The zona pellucida is a glycoprotein coat surrounding the oocyte. Changes to this layer, triggered by the action of a single sperm binding to its receptors, induce the cortical granules to release their contents. The enzymes released from the

cortical granules act to crosslink the glycoproteins of the *zona pellucida*, thus rendering it impenetrable to further sperm ***(6)***.

Cryopreservation has been shown to result in the premature release of the contents of cortical granules, thus creating a block to sperm penetration ***(7)***. Conversely, cryopreservation can lead to damage to the *zona pellucida* resulting in multiple sperm entry. Both of these problems can be overcome by application of the intracytoplasmic sperm injection (ICSI) technique, whereby a single sperm is injected into the oocyte to achieve fertilization. The microtubular spindle is the structure on which the condensed chromosomes are aligned in mature oocytes and is responsible for the movement of chromosomes during cell division. Damage to this structure can lead to aneuploidy. During cryopreservation the microtubular spindle has been shown to disassemble, although there is growing evidence that the spindle is capable of repair on warming ***(8–10)***. Whether such repair has adverse consequences during development of the resulting embryo is still uncertain. One way of avoiding the potential for damage to the microtubular spindle is to cryopreserve the oocyte before the spindle is formed, when the chromosomes are contained within the germinal vesicle and the oocyte is termed immature. However, success following cryopreservation of immature oocytes is less than that following cryopreservation of mature oocytes. If protocols for hormonal stimulation of ovaries to induce release of numerous mature oocytes are not available or not easily applicable, then only immature oocytes, contained within the ovaries, may be available for storage. The major problem with cryopreserving immature oocytes is that they must be matured in vitro in order to become fertilizable. This maturation involves communication between the oocyte and the cumulus cells that surround it ***(11)***. The cumulus cells are much smaller than the oocyte and are connected with it by numerous gap junctions. Development of cryopreservation protocols that allow survival of the oocyte, the cumulus cells, and the connections between the two is difficult ***(12)***. Also, techniques for maturation in vitro require further refinement in most species. Maturation of oocytes in vitro prior to cryopreservation may produce poor-quality oocytes that are more prone to damage during cryopreservation. As well as these general problems, the oocytes of some species have further characteristics that make them still more difficult to freeze; for example, porcine oocytes have a high lipid content and are sensitive to chilling to temperatures at or below 15°C ***(13)***.

The protocols used for the cryopreservation of unfertilized oocytes have largely been adopted from embryo cryopreservation techniques. As with embryo cryopreservation, both slow controlled-rate cooling and vitrification techniques have been successfully applied. The technique most commonly applied to the cryopreservation of human oocytes is that of slow cooling in the presence of propanediol and sucrose. This technique, combined with

ICSI, first yielded a live birth from cryopreserved human oocytes in 1997 ***(14)***. Since then, the number of human live births has been increasing steadily, with approx 100 children having been born from cryopreserved human oocytes, fertilized using the ICSI technique, by 2004 ***(9)***. Vitrification has also produced a very small number of human births ***(15,16)***. Because of differences between species, no single cryopreservation method is suitable for all species and all developmental stages of oocyte. Despite success, the proportion of oocytes surviving and going on to produce live births is still rather disappointing. Following oocyte cryopreservation for human oocytes only 1–5% of oocytes frozen result in live births. The quality of the oocytes to be preserved is a crucial factor in survival post cryopreservation ***(17)***. Counseling regarding expected outcome should be provided to patients prior to oocyte cryopreservation being offered as a treatment option.

2. Materials

2.1. Slow Controlled-Rate Cooling

1. A controlled-rate freezing machine (available from several sources such as Planer plc, Middlesex, UK or Asymptote, Cambridge, UK) set to hold at 20°C, then cool at –2°C/min to –7°C, hold at –7°C for 10 min prior to seeding (*see* **Note 1**), hold at –7°C for 10 min after seeding, then cool at 0.3°C/min to –30°C, then at –50 to –150°C and finally hold at –150°C for 10 min (*see* **Note 2**).
2. Plastic straws (*see* **Note 3**).
3. Plugs for straws (IMV, L'Aigle, France), sealing powder or heat sealer (*see* **Note 4**).
4. Pulled glass pipets or automated pipettor for transferral of oocytes.
5. Tissue culture dishes (Falcon, Becton and Dickinson Co.).
6. Dissecting microscope (×40 magnification).
7. Hotplate set at 37°C.
8. Forceps cooled in liquid nitrogen (optional; *see* **Note 1**).
9. Liquid nitrogen and liquid nitrogen Dewars, preferably at least two storage Dewars (*see* **Note 5**) and one for transporting samples to storage Dewar and for cooling of forceps, if used.
10. Sterile scissors.
11. Syringes and needle (optional; *see* **Notes 6** and **7**).
12. Heated water bath set at 30°C.
13. Safety equipment, e.g., cryogloves, face shield, and oxygen depletion monitor.
14. Heated gassed incubator (5% [v/v] CO_2, humidified atmosphere).
15. Oocyte culture medium, e.g., for human oocytes fertilization medium (Cook IVF, Brisbane, Australia), and for murine oocytes Tyrode's medium (Invitrogen, Paisley, UK).
16. Dulbecco's phosphate-buffered solution (PBS; Invitrogen) for human oocytes supplemented with 30% (w/v) protein supplement, e.g., plasma protein supplement (Baxter AG, Vienna, Austria) or serum protein supplement (Pacific Andrology,

CGA/Diasint, Florence, Italy) for mouse oocytes supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Invitrogen).

17. Freezing solutions: 1.5 *M* 1,2-propanediol (PrOH) and 1.5 *M* PrOH plus 0.2 *M* sucrose, both made up in PBS with protein supplement.
18. Thawing solutions: 1.0 *M* PrOH plus 0.2 *M* sucrose, 0.5 *M* PrOH plus 0.2 *M* sucrose, and 0.2 *M* sucrose, all made up in PBS with protein supplement.
19. Hyaluronidase (optional; *see* **Note 8**).

2.2. Vitrification

1. Plastic straws (*see* **Notes 3** and **9**).
2. Plugs for straws (IMV), sealing powder, or heat sealer (*see* **Note 4**).
3. Pulled glass pipets or automated pipettor for transferral of oocytes.
4. Tissue culture dishes (Falcon, Becton and Dickinson Co.).
5. Dissecting microscope (×40 magnification).
6. Thermocouple.
7. Device to hold straw horizontally above liquid nitrogen vapor without covering area of straw containing oocytes (*see* **Note 10**).
8. Hotplate set at 37°C.
9. Liquid nitrogen and liquid nitrogen Dewars, preferably at least two storage Dewars (*see* **Note 5**), plus a container capable of holding liquid nitrogen and accommodating a straw held horizontally.
10. Sterile scissors.
11. 3X 1-mL syringe and 2X needle.
12. Heated water bath set at 20°C.
13. Safety equipment, e.g., cryogloves, face shield, and oxygen depletion monitor.
14. Heated gassed incubator (5% CO_2, humidified atmosphere).
15. Oocyte culture medium, e.g., for human oocytes fertilization medium (Cook IVF), for murine oocytes Tyrode's medium (Invitrogen).
16. PBS for human oocytes supplemented with 30% (w/v) protein supplement, e.g., plasma protein supplement (Baxter AG) or serum protein supplement (Pacific Andrology, CGA/Diasint) for mouse oocytes supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Invitrogen).
17. Vitrification solutions: 6 *M* dimethyl sulfoxide plus 1 mg/mL polyethylene glycol (MW 8000) added to 4X strength PBS medium without $CaCl_2$ (*see* **Note 11**). The solution should be made up by adding 1% (v/v) distilled water to 4X PBS prior to adding the dimethyl sulfoxide and polyethylene glycol. Then add the protein supplement and make up almost to volume with water prior to adding $CaCl_2$. Finally, make up to volume. Keep at room temperature until required. This solution will be referred to as VSDP (*see* **Note 12**). Make up dilutions of 25 and 65% (v/v) VSDP using PBS containing protein supplement.
18. Dilution solution consisting of 1 *M* sucrose made up in PBS containing the protein supplement.
19. Hyaluronidase (optional; *see* **Note 8**).

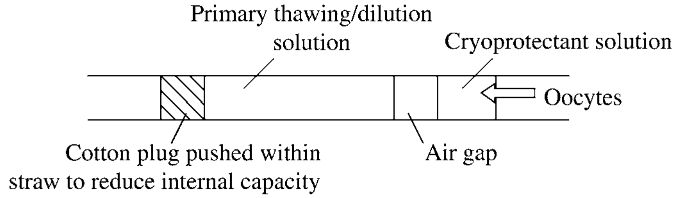

Fig. 2. Schematic diagram of straw prepared for cryopreservation of oocytes.

3. Methods

3.1. Slow Controlled-Rate Cooling

The slow controlled-rate cooling method described uses a mixture of the permeating cryoprotectant PrOH and nonpermeating sucrose. Recent studies suggest that 0.3 *M* sucrose yields better survival than 0.2 *M* sucrose in human oocytes ***(18)***. This is thought to be largely from greater dehydration of the cells prior to freezing. Optimal exposure time to PrOH plus 0.2 *M* sucrose is 5–10 min, whereas optimal exposure time to PrOH plus 0.3 *M* sucrose is yet to be determined. However, an exposure time of 2 min will give a level of dehydration equivalent to that achieved with a 5-min exposure to PrOH plus 0.2 *M* sucrose ***(19)***. A further potential modification to the method described next is the choice of medium in which to dilute the cryoprotectants. Recent studies have shown a medium in which some of the sodium has been replaced by choline to be preferable to Dulbecco's PBS medium ***(20–22)***.

1. Straws should be loaded with a 1-cm column of 1.5 *M* PrOH plus 0.2 *M* sucrose and left at room temperature (20 ± 2°C) until required (*see* **Fig. 2**; **Note 6**).
2. Place one 0.5-mL droplet each of PBS plus protein supplement, 1.5 *M* PrOH, and 1.5 *M* PrOH plus 0.2 *M* sucrose in a tissue culture dish. Label each droplet.
3. No more than five oocytes (*see* **Note 13**) should be placed in the droplet of PBS (cumulus cells may be removed, *see* **Note 8**) and then placed in the droplet of 1.5 *M* PrOH for 10 min at room temperature. The oocytes are then moved to the droplet of 1.5 *M* PrOH plus 0.2 *M* sucrose for 5 min at room temperature.
4. The oocytes are then loaded into the prepared straws within the column of 1.5 *M* PrOH plus 0.2 *M* sucrose and the straw is sealed (*see* **Note 4**).
5. The outside of the straws should be decontaminated using 70% ethanol in distilled water, an alcohol wipe, or, preferably, a less volatile disinfecting agent e.g., hypochlorite ***(23)*** and the straws are then placed immediately within a freezing machine set at 20°C. The straws should not be placed in bundles as this will affect the rate of cooling. The machine should then be cooled to –7°C at –2°C/min. Having been held at this temperature for 10 min, ice nucleation should be initiated in the solution containing the oocytes (*see* **Note 1**). The cooling regime is then resumed with the temperature being held at –7°C for 10 min to allow dissipation of the heat of crystallization. Once the cooling protocol has been completed and

the samples are held at –150°C, the straws should be decontaminated, as above, and, wearing appropriate protective equipment, placed in a liquid nitrogen storage Dewar (*see* **Note 14**).

6. Prior to warming the straws, prepare dishes containing a 0.5–1 mL droplet each of 1.0 *M* PrOH plus 0.2 *M* sucrose, 0.5 *M* PrOH plus 0.2 *M* sucrose, and 0.2 *M* sucrose and PBS with protein supplement. Label each droplet.
7. Wearing suitable protective equipment, warm the straws by holding them in air for 30 s, holding either end of the straw *not* the area containing the oocytes. Then place the straws in the water bath set at 30°C for 30 s or until the ice has just melted.
8. Decontaminate the straw. The plug, if used, should be removed from the straw or the ends of the straw should be cut with sterile scissors and the contents then expelled (*see* **Note 7**) into the dish containing 1 *M* PrOH plus 0.2 *M* sucrose.
9. The oocytes should remain in this solution for 5 min at room temperature before being moved into the droplet of 0.5 *M* PrOH plus 0.2 *M* sucrose again for 5 min at room temperature.
10. The oocytes are moved into 0.2 *M* sucrose for 10 min at room temperature.
11. The oocytes are then placed in the droplet of PBS for 20 min, 10 min at room temperature, and 10 min at 37°C on a hotplate.
12. The oocytes should then be cultured (2–3 h human, 30 min mouse) within an incubator at 37°C (5% CO_2) in a suitable culture medium to allow recovery prior to attempted fertilization (*see* **Note 15**).

3.2. Vitrification

Vitrification is advantageous over slow controlled-rate cooling in that expensive cooling machines are not required and the technique can be performed in the field. However, the need for high concentrations of cryoprotectant means that the times stated for cryoprotectant exposure must be strictly adhered to and manipulation of oocytes in these highly viscous solutions is technically demanding. The vitrification method described has resulted in high blastocyst formation ***(24)*** and live births ***(25)*** in mice, although the technique is prone to variability ***(26)***. The cryoprotectant mixture and cryopreservation vessel could be modified but the basic techniques described are applicable to all vitrification protocols.

1. Straws should be loaded with an approx 0.5-cm column of 100% VSDP (*see* **Fig. 2**; **Note 6**). Take care not to wet the sides of the straw with sucrose (*see* **Note 16**). Leave prepared straw at room temperature until required.
2. Place one 50-µL droplet each of 25, 65, and 100% (v/v) VSDP in a tissue culture dish. Label each droplet.
3. Place liquid nitrogen to a level equivalent to at least the length of the straw into the vessel capable of accommodating the straw horizontally. Use the thermocouple to determine the point above the liquid nitrogen at which the temperature is –140°C and temporarily affix the thermocouple at this level.

4. Pipet a maximum of five oocytes (*see* **Note 13**) into a 25% VSDP droplet and leave at room temperature for 3–5 min.
5. Transferring as little of the solution as possible (*see* **Note 17**), pipet the oocytes from the 25% (v/v) VSDP into a 65% (v/v) VSDP droplet.
6. As quickly as possible, move the oocytes into a droplet of 100% VSDP.
7. Immediately draw up a small amount of 100% VSDP into a glass pipet and collect the oocytes. Transfer the oocytes into the column of 100% VSDP contained within the prepared straw.
8. Seal the open end of the straw (*see* **Note 4**) and decontaminate its surface with 70% (v/v) ethanol, an alcohol wipe or, preferably, a less volatile disinfecting agent (e.g., hypochlorite) ***(23)***. Hold the straw using a holder and, wearing suitable protective equipment, immediately position the straw horizontally at the position above the liquid nitrogen at which the temperature is –140°C (*see* **Note 18**). Keep the straw in this position for 3 min and then plunge the straw into liquid nitrogen.
9. Transfer the straw to a liquid nitrogen storage vessel (*see* **Note 14**).
10. Prior to warming the straw, place 1 mL of 1 *M* sucrose solution in a culture dish, half fill a 1-mL syringe with 1 *M* sucrose, and place two 50-µL droplets of sucrose and two 50-µL droplets of PBS with protein supplement in a culture dish. Label the droplets.
11. Wearing suitable protective equipment, remove the straw from liquid nitrogen storage. Hold the straw in air for 10 s, holding either end of the straw and *not* the area containing the oocytes, then plunge the straw into the water bath at 20°C for 10 s.
12. Decontaminate the straw, as in **step 8**, and cut through it using sterile scissors in the area containing the sucrose. Remove the plug from the other end, if used, or cut with sterile scissors. Attach the syringe containing sucrose to one end of the straw and hold the other end over the dish containing 1 mL sucrose. Flush the contents of the straw and syringe into the dish and ensure good mixing.
13. Immediately begin to look for the oocytes using the dissecting microscope (*see* **Note 19**) maintaining the oocytes at room temperature throughout. As soon as the oocytes are located place them in one of the droplets of sucrose.
14. Immediately move the oocytes into the second droplet of sucrose at room temperature. When the oocytes have been in contact with 1 *M* sucrose for a total of 5 min, transfer the oocytes into a droplet of PBS and leave for 10 min at room temperature. Move the oocytes into the second droplet of PBS and leave them for 10 min on the hotplate.
15. The oocytes should then be placed in oocyte culture medium, within an incubator (2–3 h human, 30 min mouse), prior to attempted fertilization (*see* **Note 15**).

4. Notes

1. Seeding is the initiation of ice nucleation. This can be achieved automatically in some freezing machines, or can be initiated manually by removing the straws from the machine and touching the solution with forceps that have been precooled in liquid nitrogen.

2. Samples should be cooled below the glass transition temperature (–130°C) within the cooling machine. Warming above this temperature during transfer to storage and during storage should be avoided because only below this temperature will all biological activity cease and the samples be safe from deterioration.
3. Oocytes can be slow cooled within cryovials or straws. The poor thermal conductivity properties and possibility of vial leakage makes straws the preferred option. Cryovials are not suitable for vitrification. Straws are available from IMV, Minitub, (Tiefenbach, Germany), and CryoBioSystem (France). The use of high-security straws is recommended because they are heat sealable, guaranteed leak-proof to given pressures, shatterproof, bacteria/virus proof, and tested extensively to verify nontoxicity ***(27)***. Also, straws are available with two compartments, allowing identification information to be stored within the straw itself thereby reducing the risk of tampering.
4. Straws can be sealed by heating the ends, although care must be taken not to heat the solution containing the oocytes, or to damage the straw by creating stress fractures that can crack on cooling and allow contamination of the sample or liquid nitrogen entry that risks explosion on warming. Alternatively, straws can be sealed by insertion of plugs or sealing powder, both of which should be wet in order to ensure a good seal.
5. Ideally, oocytes should be stored individually within straws and the straws kept in at least two different locations so that should an accident occur with one straw then all is not lost. In areas of high risk, storage on two sites may be advisable. Samples from patients who have been screened, those who are unscreened, and those with known pathogens should be stored in separate storage tanks. Accurate records should be kept of the location of the straws within each storage vessel.
6. The straw can be filled using a small syringe and needle. Pushing the cotton plug along the straw will reduce the capacity of the straw (*see* **Fig. 2**). The plug should be wetted as this will help prevent liquid nitrogen entry. If the plug is wetted by insertion of a sizeable column of the primary thawing/dilution solution, then expulsion of this solution along with the oocytes following thawing will aid dilution of the cryoprotectant. An air gap should be left between this section and the area to contain the oocytes—a column of cryoprotectant inserted using a fresh syringe and needle. Straws should be clearly labeled, with an appropriate marker, i.e., one that will not smudge or be erased during storage.
7. The straw contents can be expelled by inserting a 1-mL syringe containing at least 0.5 mL of primary thawing/dilution solution and expelling the contents of both the straw and syringe into a dish.
8. Studies have been performed with oocytes that have been denuded of cumulus cells (by means of gentle pipetting and/or treatment with hyaluronidase) prior to freezing or frozen with the cumulus intact. No clear advantage either way is evident.
9. Faster cooling/warming rates can be achieved and the risk of ice crystal formation reduced by using straws that have been pulled to reduce the wall thickness, or by use of such devices as nylon loops or microscope grids.

10. A piece of plastic that can be inserted into a straw and is approximately twice the length of a straw is ideal. The plastic is inserted into the end of the straw and then bent through 90°. The straw can then be placed close to the nitrogen in the horizontal position and the holder can be held by hand at a safe distance from the nitrogen.
11. Addition of the high concentrations of cryoprotectant used in vitrification solutions directly to the PBS medium will result in reduction of the salt concentrations within that medium. Using a concentrated PBS medium allows the cryoprotectant to be added prior to the addition of water to give the required volume of single-strength PBS medium.
12. The vitrification solution could be replaced with other combinations of cryoprotectant, for example, ethylene glycol together with sucrose, often in combination with a macromolecule such as Ficoll, which has been used successfully for the preservation of bovine and human oocytes. Exposure to this cryoprotectant mixture can be performed at 37°C, thereby avoiding any cooling-related damage.
13. In order to adhere to the timing of each step of the procedure, oocytes should be processed in small batches.
14. It should be noted that straws allow rapid heat transfer and hence are susceptible to temperature change during handling, for example on transfer from the cooling machine to liquid nitrogen for storage and during handling for identification purposes prior to thawing. Auditing of cryopreserved samples should only be performed after careful consideration of the risks of warming the samples. Both liquid nitrogen and liquid nitrogen vapor should be considered to contain pathogens. Straws that are not intact should be discarded. Care should be taken to prevent the bending of straws, which may lead to cracks and entry of contaminants from the liquid nitrogen, as well as a risk of explosion on warming as liquid nitrogen within the vessel expands. If storing in the vapor phase, the level of liquid nitrogen should be carefully monitored to ensure stability of storage temperature. Alarms and automatic filling systems are available, but it is recommended that the level of liquid nitrogen still be checked manually at regular intervals.
15. There is evidence to suggest that the microtubular spindle is capable of repair during a period of culture post-thaw.
16. The presence of the sucrose solution on the sides of the straw would dilute the concentration of cryoprotectant in the VSDP column and, more importantly, may allow the propagation of ice crystals from the sucrose solution into the area within which the oocytes are contained.
17. Care must be taken not to dilute the cryoprotectant concentration in these small droplets, as the use of small droplets aids location of the oocytes in such viscous solutions.
18. The straw is held at –140°C just below the glass transition temperature, rather than plunging directly into liquid nitrogen in order to reduce the occurrence of cracks in the vitrified glass which, on warming, may form sites of ice nucleation. It is important to measure the temperature, as extreme fluctuations in temperature are present across a few centimetres in liquid nitrogen vapor.

19. Oocytes are particularly difficult to locate in this solution. The oocytes must spend no longer than 5 min in sucrose at this point and this time can pass quickly.

References

1. Whittingham, D. G. (1977) Fertilization in vitro and development to term of unfertilized mouse oocytes previously stored at –196. *J. Reprod. Fertil.* **49,** 89–94.
2. Al-Hasani, A., Kirsch, J., Diedrich, K., Blanke, S., van der Ven, H., and Krebs, D. (1989) Successful embryo transfer of cryopreserved and *in vitro* fertilised rabbit oocytes. *Hum. Reprod.* **4,** 77–79.
3. Fuku, E., Kojima, T., Shoiya, Y., Marcus, G. J., and Downey, B. R. (1992) *In vitro* fertilization and development of frozen-thawed bovine oocytes. *Cryobiology* **29,** 485–492.
4. MacLellan, L. J., Carnevale, E. M., da Silva, M. A. C., Scoggin, C. F., Bruemmer, J. E., and Squires, E. L. (2002) Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. *Theriogenology* **58,** 911–919.
5. Chen, C. (1986) Pregnancy after human oocyte cryopreservation. *Lancet* **1,** 884–886.
6. Bleil, J. D. and Wassarman, P. M. (1980) Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Develop. Biol.* **76,** 185–202.
7. Carroll, J., Depypere, H., and Matthews, C. D. (1990) Freeze-thaw induced changes of the zona pellucida explains decreased rates of fertilisation in frozen-thawed mouse oocytes. *J. Reprod. Fertil.* **90,** 547–553.
8. Bianchi, V., Coticchio, G., Fava, L., Flamigni, C., and Borini, A. (2005) Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. *Hum. Reprod.* **20,** 1078–1083.
9. Stachecki, J. J. and Cohen, J. (2004) An overview of oocyte cryopreservation. *Reprod. Biomed. Online* **9,** 152–163.
10. Rienzi, L., Martinez, F., Ubaldi, F., et al. (2004) Polscope analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. *Hum. Reprod.* **19,** 655–659.
11. Brower, P. T. and Schultz, R. M. (1982) Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. *Dev. Biol.* **90,** 144–153.
12. Ruppert-Lingham, C. J., Paynter, S. J., Godfrey, J., Fuller, B. J., and Shaw, R. W. (2003) Developmental potential of murine germinal vesicle stage cumulus-oocyte complexes following exposure to dimethylsulphoxide or cryopreservation: loss of membrane integrity of cumulus cells after thawing. *Hum. Reprod.* **18,** 392–398.
13. Isachenko, V., Soler, C., Isachenko, E., Perez-Sanchez, F., and Grischenko, V. (1998) Vitrification of immature porcine oocytes: effects of lipid droplets, temperature, cytoskeleton, and addition and removal of cryoprotectant. *Cryobiology* **36,** 250–253.

14. Porcu, E., Fabbri, R., Seracchioli, R., Ciotti, P. M., Magrini, O., and Flamigni, C. (1997) Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. *Fertil. Steril.* **68,** 724–726.
15. Kuleshova, L., Gianaroli, L., Magli, C., Ferraretti, A., and Trounson, A. (1999) Birth following vitrification of a small number of human oocytes. *Hum. Reprod.* **14,** 3077–3079.
16. Yoon, T. K., Chung, H. M., Lim, J. M., Han, S. Y., Ko, J. J., and Cha, K. Y. (2000) Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated *in vitro* fertilization-embryo transfer program. *Fertil. Steril.* **74,** 180–181.
17. Paynter, S. J. (2000) Current status of the cryopreservation of human unfertilised oocytes. *Hum. Reprod. Update* **6,** 449–456.
18. Fabbri, R., Porcu, E., Marsella, T., Rocchetta, G., Venturoli, S., and Flamigni, C. (2001) Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Hum. Reprod.* **16,** 411–416.
19. Paynter, S. J., Borini, A., Bianchi, V., De Santis, L., Flamigni, C., and Coticchio, G. (2005) Volume changes of mature human oocytes on exposure to cryoprotectant solutions used in slow cooling procedures. *Hum. Reprod.* **20,** 1194–1199.
20. Stachecki, J. J., Cohen, J., and Willadsen, S. M. (1998) Cryopreservation of unfertilized mouse oocytes: the effect of replacing sodium with choline in the freezing medium. *Cryobiology* **37,** 346–354.
21. Quintans, C. J., Donaldson, M. J., Bertolino, M. V., and Pasqualini, R. S. (2002) Birth of two babies using oocytes that were cryopreserved in a choline-based freezing medium. *Hum. Reprod.* **17,** 3149–3152.
22. Boldt, J., Cline, D., and McLaughlin, D. (2003) Human oocyte cryopreservation as an adjunct to IVF-embryo transfer cycles. *Hum. Reprod.* **18,** 1250–1255.
23. Mortimer, D. (2004) Current and future concepts and practices in human sperm cryobanking. *Reprod. Biomed. Online* **9,** 134–151.
24. O'Neil, L., Paynter, S. J., Fuller, B. J., and Shaw, R. W. (1997) Vitrification of mature mouse oocytes: improved results following addition of polyethylene glycol to a dimethyl sulfoxide solution. *Cryobiology* **34,** 295–301.
25. O'Neil, L., Paynter, S. J., Fuller, B. J., and Shaw, R. W. (1999) Birth of live young from mouse oocytes vitrified in 6 M dimethyl sulfoxide supplemented with 1 mg/ml polyethylene glycol. *Cryobiology* **39,** 284.
26. Fahy, G. M., Wowk, B., Wu, J., and Paynter, S. J. (2004) Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology* **48,** 22–35.
27. Mortimer, D. (2004) Setting up risk management systems in *in-vitro* fertilization laboratories. *Clinical Risk* **10,** 128–137.